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Effect of bioremediation or organic treatments of soil contaminated with some heavy metals on the biological and L- glutaminase activity

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For the purpose of identify the ability of bioremediation or organic treatments of soil to reduce the harmful effect of heavy metals including Zn, pb and cd on the biological activity such as soil respiration, the total number of fungi and the activity of the L-glutaminase enzyme have been studied. A laboratory incubation experiment was applied in the Soil Microbiology Laboratory in the Department of Soil Sciences and Water Resources in the College of Agriculture– Basrah University. The soil of the surface layer (0-30 cm) from Qurna district, north of Basrah province was contaminated with the critical levels of the heavy metals cd, Pb, Zn and were placed in an incubator at 30°C for 14 days. The biological and organic treatments process was applied by inoculating soil contaminated with *Aspergillus niger* or humic acid (40 L ha^{-1}) or sterilized cow manure (4%), incubated at 25°C for 30 days, then some biological parameters were estimated at the periods of 10, 20 and 30 days. Results have been showed that an inhibitory effect of the studied heavy metals on the biological parameters, and the Cd has been recorded the highest percentage of inhibition of total number of fungi (30.67%), the amount of CO_2 released (13.93%) and the activity of the L-glutaminase enzyme (45.33%) compared to the control treatment. The ability of the treatments materials to remove heavy metals were (89.02%) for *A. niger* compared to humic acid (63.48%) and cow manure (48.71%). It was reflected in an increase in the number of fungi, which recorded 152, 167.75, 174.5 cfu g^{-1} when treated with *A. niger*, cow manure and humic acid, respectively compared to the control treatment which was (65.92 cfu g^{-1}). The biological and organic treatments also increased the level of liberated CO_2 from the polluted soil to 268.62, 253.86, 303.59 mg CO_2 g^{-1}, respectively compared to the control treatment. The activity of the L-glutaminase enzyme has increased due to application of treatments. It was 57.02, 48.97, 70.41 µg NH_4^+ g^{-1} 2hr.$^{-1}$ for inoculated with *A. niger* or treated with cow manure and humic acid, respectively, while the control treatment was 37.19 µg NH_4^+ g^{-1}2hr.$^{-1}$.

Keywords: Bioremediation, Heavy metals, fungi, humic acid, L-glutaminase, Qurna district, aspergillus nige, Humic acid.

INTRODUCTION

In recent years, soil pollution is one of the most prominent, complex and difficult problems to solve. The development of the global economy has led to an increase in soil pollution, especially with heavy metals. It is colorless and odorless and difficult to notice. Studies estimated that more than 5 million hectares of land which hazardous minerals exceed their critical limits in the soil worldwide (Giller *et al*., 2017). Heavy metals are natural substances that have an atomic number higher than 20. They are described as having a relatively high density (at least 5 g cm^{-3}). They are considered toxic even in their low concentrations due to their stability, long half-life and the possibility of bioaccumulation (Nath *et al*., 2019). It poses an issue to the soil, plants and human health through its transmission through the food chain without changing its properties, and the damages resulting from it may not appear directly or for short periods. However, when environmental conditions change, these minerals have become active in the soil and cause serious damage, including a decrease in the activities of soil enzymes (Meng *et al*., 2018).

High levels of these elements create environmental problems and affect the growth and activity of microorganisms. As well as, they can damage cell membranes and disrupt enzymatic and cellular functions by blocking the active sites of enzymes forming complexes with protein molecules and making them inactive. These effects disrupt the microbial cell membrane and destroy the entire cell (Vodyanitskii, 2016). In Iraq, heavy metals are released into the environment through natural sources that are affected by environmental factors leading to the release of these elements. The soils of Basrah province in

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southern Iraq have been exposed to many sources that release heavy metals, such as oil industries and fuel refineries that add tetraethyl led to fuel for improving it and increase its octane number in addition to the most important ports in which transport traffic and oil spills are active. As well as, the excessive use of fertilizers and pesticides for increasing soil fertility and production in quantity and quality. Furthermore, the region has been exposed to explosives due to the wars during the past years. The metals including pb, Cd and Zn are the most pollutants present in the soil and waters of such region.

The lead (pb) is one of the most important and common pollutants in the world, and high levels of it pose a great harm to human health (Sparks, 2005). The toxicity comes as a result of the change in the composition of nucleic acids, the inhibition of enzymatic protein and cell wall dysfunction (Bruins *et al*., 2000). Cadmium (Cd) is one of the most chemical pollutants due to its cumulative naturally and its toxic effects on all living organisms. Its concentration in the environment does not decrease, but rather changes from one form to another (Schouw *et al*., 2002). It is an unnecessary element in biological processes including microorganisms, plants, and animals. The International Agency for Research on Cancer considered it carcinogenic to humans (WHO, 2004). It accumulates in the soil due to the use of liquid industrial waste, phosphate fertilizers, and agricultural pesticides. The danger of this element is the ability to form complexes with biomolecules. It attains its toxic properties from that the chemical structure similar to Zn, which is one of the trace nutrients that needed by microorganisms and plants. The Zn is widespread in nature and is not found free, but combined with other elements. It is one of the reducing agents in chemical reactions and is considered one of the beneficial elements in nature, but it is a toxic element at higher concentrations than the limits recommended for human health. Its toxicity due to its interference with the absorption of essential elements, especially phosphorus, magnesium, copper and iron (Dowdy and Volk, 1983).

Different methods have been used to remove heavy metals from the soil, such as chemical and physical, but they may be relatively expensive and not suitable for large areas of land. Bioremediation is considered one of the safest, cleanest, most effective and environmentally friendly technologies for disinfecting contaminated sites. It is also inexpensive and does not produce secondary toxic. In the environment, different organisms such as bacteria, yeasts, fungi, algae, and plants have used as key tools in processing heavy metals (Alloway, 2013). Microbial treatment is a form of biological treatment of contaminated soil that uses fungi and bacteria to increase biological and enzymatic activity to reduce the pollutants or reduce their negative impact by using different mechanisms such as adsorption, precipitation, or oxidation and reduction of heavy metal ions in the soil (Gui *et al*., 2023). Humic acids are one of the materials that used to reduce the harmful effects of heavy metals. One of the most important features of these materials is that they have high negative charges because they contain function groups of carboxyl and hydroxyl within phenols and alcohols. They also contain carbonyls and amino acids that enable them to interact with mineral substances and pollutants in the soil through an oxidation-reduction reactions, complexes and chelation as well as formation of bonds with positive ions and water in the soil (Sharma *et al*., 2021). Humic materials are considered a suitable environment aspect for soil microorganisms. They can reduce pH of the alkaline soils and reduce salinity. Also, humic acids and organic matter are of great importance in improving the vital characteristics of the soil, which is represented in encouraging the growth of soil microorganisms because of their high concentration of carbon. It has a major role in the biotic and abiotic interactions that occur in the plant roots, which reflect positively on different biochemical reactions, including the activities of soil enzymes, enzyme stabilization and preservation by organic colloids, and sequestration by humic (Gopinath et *al*., 2020). There are few studies in the soils of southern Iraq related to biological soil reclamation. The study aim was to reduce the negative impact of heavy metals on the biological activity by using organic materials or fungi isolated from the soil of the study.

MATERIALS AND METHODS

Random samples of soil (0-30 cm) of Qurna district, north of Basrah province were mixed to form a composite sample, then placed in sterilized polyethylene bags. Part of sample was air dried, ground and passed through a sieve with a diameter of 2 mm to perform preliminary analyzes of the physical, chemical and biological properties of the soil (Table 1). The rest of the soil was kept at a temperature of 4°C for subsequent biological experiments.

Table 1. Some chemical, physical and biological properties of the studied soil

Traits	Units	Values
pH		7.84
EC	dS m^{-1}	4.5
CEC	$Cmol^{(+)}$ kg^{-1}	18.6
CaCO3	g kg^{-1}	210.4
Organic matter	g kg^{-1}	6.4
Total nitrogen	g kg^{-1}	1.1
Available nitrogen	g kg^{-1}	0.021
Available phosphorous	g kg^{-1}	0.0542
Total fungi	Cfu g^{-1}	15×10^3
Clay	g kg^{-1}	458
Silt	g kg^{-1}	326
Sand	g kg^{-1}	216
Soil texture		Silty loam

500 g of soil was contaminated with cadmium, lead and zinc salts in the form of elemental sulphate with a critical concentration of 100, 100, 3 ppm respectively (Kloke, 1980), while maintaining the control treatment without heavy metals. All samples were incubated at a temperature of 30°C. One-third of the above treatments were inoculated with *Aspergillus niger* (isolated from the soil of the same experiment, grown and identified on Potato dextrose agar (PDA) and purified several times using sub cultures in the same nutrient medium and diagnosed according to Klich (2002) at a rate of 10 ml of inoculum per 100 g of soil with a population density of 50 × 10^3 cfu. The other third was treated with sterilized cow manure at rate of 4% based on dried soil, while the last third was treated with 20 L (ha^{-1}) of humic acid (HA). HA was extracted from composted cow manure by 0.1 *N* NaOH, removing the precipitate (humin) and treating the filtrate with concentrated HCl until the pH reached about 2 and left until the next day to form a precipitate that represents humic acid (Page *et al*, 1982).

The treatments were moistened to 70% of the field capacity and incubated at 25°C for 30 days. The moisture was maintained by compensating the weight difference with sterile distilled water, taking into account the stirring of the soil for aeration during the experiment period. The vital characteristics were estimated after 10, 20 and 30 days of incubation, and replicated three times which included:

1- Total number of fungi: by plate count method using PDA medium and incubation at 25°C for 5 days according to Page *et al*. (1982).

2- Soil respiration by measuring the amount of CO_2 liberated from soil treatments by absorbing it with 1*N* NaOH and then reverses denaturation with 1N HCl after adding barium chloride and phenolphthalein (Ciardi and Nannipieri *et al*., 1990). The amount of CO_2 was calculated from the equation:

meq CO_2 = meq (NaOH) – meq (HCl)

mg CO2 = meq CO2 ×eq. wt.

3- L-glutaminase activity was measured after 30 days of incubation according to Frankenberger and Tabatabai, (1991).

4- The percentage of heavy metal removal: The concentrations of heavy metals were estimated using an atomic absorption spectrophotometer according to Lindsay and Norvel (1978). The percentage of the efficiency of the treatments in removing heavy metals was calculated according to:

Heavy metal removed percentage = (initial available metal concentration–final available metal concentration) / initial metal concentration) x 100

***Statistical analysis*:** Analysis of variance (ANOVA) was used to evaluate the effect of the two factors: Heavy metals and biological or organic treatments and their interactions on number of fungi, CO_2 evoluted and L- glutaminase activity using SPSS ver. 11.0 program. Means were using Revised Least Significant Differences (RLSD) test at a significance level of 0.05.

RESULTS AND DISCUSSION

***Total number of fungi (cfu g^{-1} soil)*:** The treatment with heavy metals (Zn, pb, Cd) led to a significant decrease in the total number of fungi compared to the control treatment by 30.67, 28.33 and 19.83% for the heavy metals, respectively (Figure 1A). It is noted that Cd was the most affected metal inhibiting the total number of fungi (120.92 cfu g^{-1} soil) compared to Zn, which has given the least inhibition to the number of fungi (139.83 cfu g^{-1} soil). Cadmium attaines its toxic properties from its chemical composition similar to Zn, which is one of the trace elements needed by microorganisms and plants to replace it. Cadmium also can form strong complexes with biomolecules by linking heavy metals with proteins and peptides (Ahmed, 2012). The reason for the increase in fungal growth that treated with Zn compared to Cd and Pb may be the fact that Zn is an essential nutrient at low concentrations. It is included in the enzymatic protein synthesis and has a negative effect at high concentrations and leads to the formation of multiple biological complexes with DNA and RNA (Kozlowski *et al*., 2009). However, lead (Pb) inhibits catalytic and antibiotic interactions and reduces the protein structure of microorganisms, and inhibits the functions of the cell wall and oxidative phosphorylation (Bruins *et al*., 2000).

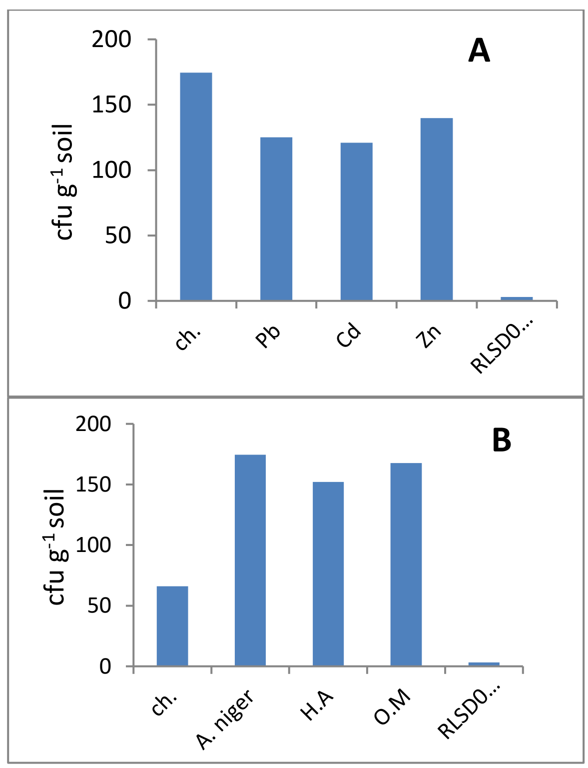

Figure 1. Effect of heavy metals, biological or organic treatment on the total number of fungi (cfu g^{-1}×10^{-3}).

Fig. 1B has been showed that there is a significant increase in the number of total fungi in soil contaminated and inoculated with *A. niger*, compared to the control treatment, with an increase of 164.715%. It is also noted that there are significant differences between the biological treatment (bioremediation) and the organic treatments (HA or cow manure) in the number of total fungi, with a significant superiority of the bioremediation with an increase of 14.8% and 4.024% compared to humic acid and cow manure, respectively. The reason for that the fungal inoculation contributed to promoting growth and increasing the total fungal numbers, in addition to the presence of *A. niger* fungus originally in the soil, and upon inoculation, its numbers has increased compared to the control treatment. Also, this fungus is a filamentous fungus that has the ability to absorb heavy metals from the environment.

The binding capacity of the elements is due to the complex cell wall of the fungus consisting of chitins, glucans, inorganic ions, lipids, nitrogen containing polysaccharide and proteins that can tolerate and detoxify heavy metals by active uptake (Annadurai *et al*., 2019). It is also found that the organic treatment also led to an increase in the number of fungi compared to the control treatment, with an increase of 130.583% and 154.475% for the two treatments of humic acid and cow manure, respectively (Figure 1B). This is because the organic matter that added to the soil provides a source of energy and food for microorganisms because it contains a high content of carbon, nitrogen, phosphorus, and other nutrients necessary for the growth of fungi and the biosynthesis of their tissues (Singh *et al*., 2018). Furthermore, to improve the physical and chemical properties of the soil that promotes the growth of fungi and increases their number (Yami and Shakya, 2005).

The interaction between treatments and heavy metals had a significant effect on the total number of fungi (Table 2). The soil inoculated with fungi and uncontaminated with heavy metals recorded the highest number of total fungi (223.33 cfu g^{-1} soil), while the lowest number of fungi was found in soil contaminated with Cd without any treatment (51.33). It is noted that a decrease in the total numbers of fungi in soil contaminated with heavy metals, whether treated or not treated biologically and organically, because heavy metals inhibit the growth of fungi and their spores, which reduces their number in the soil (Ano *et al*., 2012).

Table 2. The interaction effect between treatments and heavy metals contamination on the total number of fungi (cfu g^{-1} soil × 10^{-3}).

Sources	Zn	Cd	Pb	Ch.
Ch.	68.00	51.33	62.33	82.00
A. niger	173.67	148.00	153.00	223.33
H.A	148.33	130.33	135.33	194.00
O.M	169.83	154.00	149.33	198.33
RLSD 0.05= 2.8				

***The total CO_2 released (mg CO2 kg^{-1} soil)*:** Figure (2A) has been indicated a significant decrease in the amount of CO_2 released from the soil contaminated with heavy metals compared to the uncontaminated soil. The greatest decreasing obtained in the Cd treatment with an inhibition rate of 13.93% compared to the control treatment. The Cd is one of the most toxic heavy metals on living organisms and therefore release of CO_2 as a result of its respiration and decomposition of soil organic matter. This effect has been attributed to the large movement of Cd in the soil and its lower affinity with soil colloids.

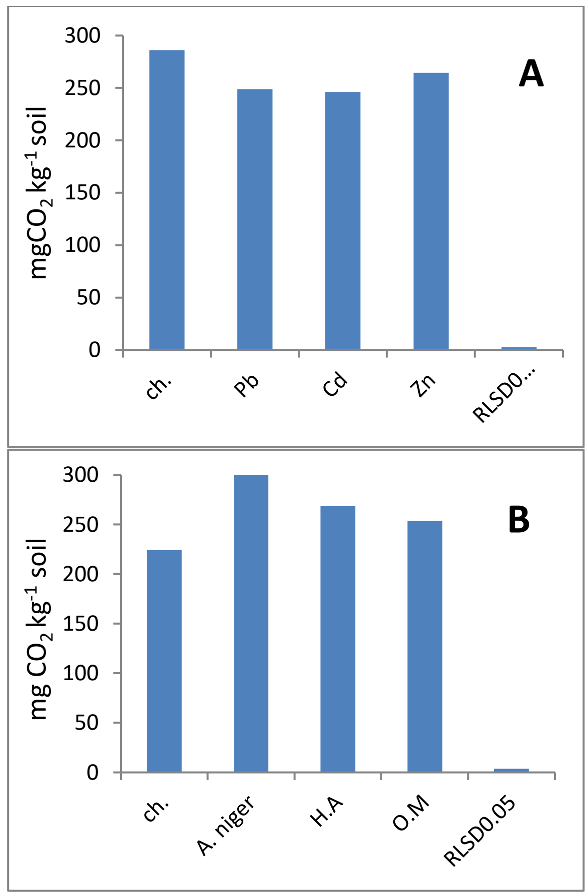

Figure 2. Effect of heavy metals, biological or organic treatment on the amount of total CO_2 released (mg CO_2 Kg^{-1} soil).

It was be found that the lowest inhibition percentage of CO_2 released when soil treated with Zn (7.6%) compared to the control treatment. The inhibition percentages ranged for the heavy metals studied in the order Cd > Pb > Zn. It is noted from (Figure 2B) that there is a significant increase in the amount of CO_2 liberated in soil contaminated with heavy metals when inoculated with *A. niger* fungus or treated with humic acid or organic matter (cow manure) with percentage of 35.31, 19.72, 13.14% for A. niger, HA and cow manure,

respectively. The fungal inoculation has showed the highest amount of CO_2 released (224.373 mg CO_2 kg^{-1} soil), this may be due to the fungal inoculation, the adaptation of these fungi in environmental media contaminated with heavy elements (Kumar *et al*., 2014). As well as, the *A. niger* is one of the fungi with a high ability to absorb heavy metal ions in a high pH range (Wasay, 1998). It is clear from figure 2B that the effect of adding humic acid is also important in increasing the bioactivity of soils contaminated with heavy metals. This may be due to the fact that this organic acid contains many active groups represented by carbonyls, carboxylates, and amides (Nannipieri *et al*., 1979). It can adsorb heavy metal ions and reduce their harmful effect on microorganisms and their effectiveness.

This also was obtained at organic matter, but its effect is less due to its need more time for mineralization and decomposition. The participation of its effective aggregates in the adsorption and chelation of heavy metals and the removal of its negative impact on soil microorganisms (Singh *et al*., 2018). Humic acid and cow manure are among the most important sources of energy, carbon and nutrients that organisms need for growth and efficiency, thus increasing the released CO_2 compared to the control treatment. The combined between the biological treatment and the heavy elements had a significant effect on the amount of CO_2 released (Table 3), with highest rate at treatment including Zn and inoculated with *A. niger* fungus (305.72 mg CO_2 kg^{-1} soil). This result is consistent with Tahir *et al*. (2017), who indicated that A. *niger* is the most fungal species contributing to the removal of heavy metals through the absorption and adsorption of ions by its biomass. So, it reduce the negative effect of these metals on the growth and effectiveness of fungi and other soil organisms. However, the lowest rate of the amount of CO_2 released was found when soil treating with Pb only without biological or organic treatment (200.14), The reason for this decrease may be that the heavy metals inhibit the growth and germination of fungal spores and the growth of bacteria. It is known that microorganisms play a major role in vital activity by decomposing organic matter and in the respiration of soil microorganisms (Ano *et al*., 2012).

Table 3. The interaction effect between treatments and heavy metal contamination on the amount of released CO_2 (mg CO_2 kg^{-1} soil).

Sources	Zn	Cd	Pb	Ch.
Ch.	223.63	210.12	200.14	263.60
A. niger	305.72	289.21	279.32	320.12
H.A	271.36	250.33	266.52	286.26
O.M	256.63	235.24	249.24	274.31
RLSD0.05= 2.8				

***Heavy metal removal percentage (%)*: ** Figure (3A) has showed the removal percentages of the studied treatment to remove heavy metals, compared to the control treatment. The superiority of inoculation with *A. niger* fungus with a removal rate of (89.02%) compared to the organic treatments with an increase of 40.23 and 82.76% compared to humic acid and cow manure, respectively. The fungi are considered the most suitable microorganisms for bioremediation due to ability of their mycelium mass to hold all kind of elements (Sanyaolu, 2018). Fungi may also produce vacuoles in which metal ions are collected and hidden by chelation. The production of melanin and other pigments that work on the active transport of ions outside the cell and the production of complex compounds resulting from the association of elements with components inside the cell (Singh *et al*., 2018).

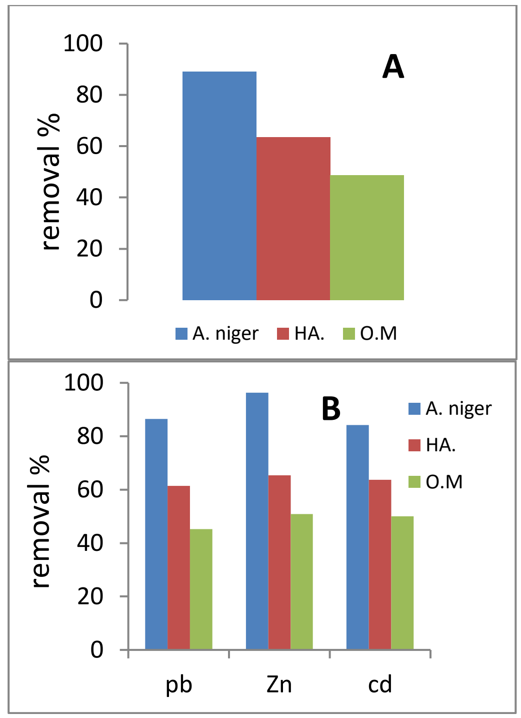

Figure 3. Effect the biological or organic treatments on the heavy metal removal percentage (%).

It is noted from Figure 3B, that the largest removal percentage was found in the soil treated with zinc and inoculated with *A. nigeria* (99.33%). This may be due to the microbiological using several strategies to survive and grow in the presence of heavy metals and remove their toxicity from the polluted environment, such as absorption, adsorption, ion exchange, complexation and entrapment in extracellular capsules, precipitation and transport across the cell membrane (El- Said *et al*., 2016; Gopinath *et al*., 2020).

***L-glutaminase activity (μgNH^{+}_{4}-N g^{-1} $2hr^{-1}$)*: ** The contamination with heavy elements (Cd, Pb, and Zn) led to a significant decrease in the activity of L-glutaminase by a decrease percentage of 45.33, 31.93, and 39.62% for Cd, Pb and Zn, respectively, compared to the control treatment (Figure 4A). Cadmium recorded the lowest activity of the

enzyme (41.243 µg NH^{+}_{4} - N g^{-1} $2hr^{-1}$), while the highest activity was associated with zinc (less inhibition). This result is consistent with the effect of heavy metals on the total number of fungi (Figure 1A). The activity of soil enzymes has a negative correlation with the content of heavy metals (Meng *et al*., 2018).

The heavy metals inhibit the enzymatic activity in several ways, such as destroying the catalytic active groups and changing the nature of the enzymatic protein (denaturation). In addition, the heavy metal ions may composite the substrate during the formation of enzyme – substrate complexes (E-S complex; Bottomley *et al*., 2020). Krajewska (2009) has been showed that heavy metals inhibit enzymatic activity by affecting the structural of enzymes and forming bonds with -SH groups in the active site. It was found that the negative effect of cadmium was greater than that of lead. The toxicity of Pb comes as a result of the change in the structure of nucleic acids and the inhibition of the enzymatic protein when lead interacts with the sulfur groups in the protein and inhibits their functions. It leads to disruption of the functions of the cell wall and oxidative phosphorylation (Bruins *et al*., 2000; Adamo *et al*., 2006).

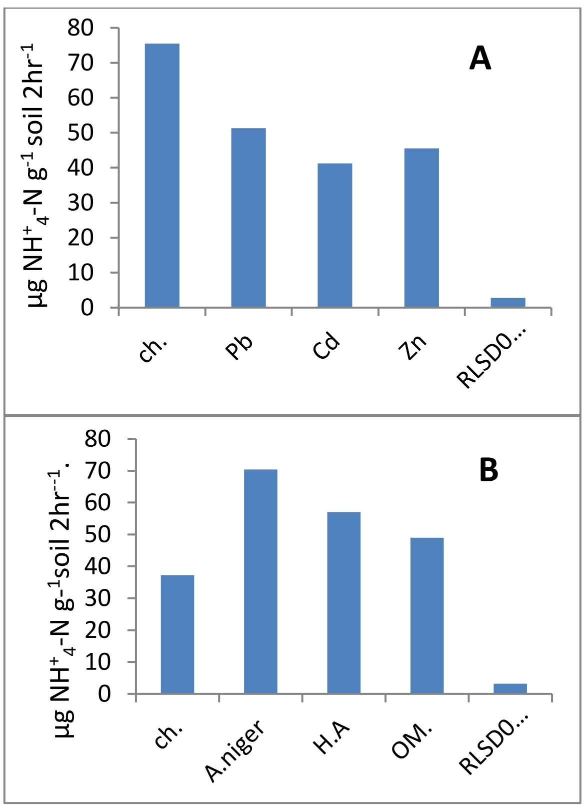

Figure 4. Effect of heavy metals, biological or organic treatment on L-glutaminase activity.

Figure (4B) indicated that the biological and organic treatments of soil contaminated with heavy metals led to a significant increase the activity of L-glutaminase and reduced the negative effect of heavy metals by an increase percentages of 89.33, 53.30 and 31.68% compared to the control treatment for *A. niger* , humic acid and cow manure, respectively. The biological treatment with *A. niger* recorded a significant increase in enzyme activity compared to humic acid and cow manure. It has indicated that the ability of fungus to use vital mechanisms represented by removing heavy metals from the medium and accumulating in fungus cells (Utobo and Tewari, 2015). In addition, *A. niger* is one of the most enzyme-producing fungal species in culture media (EL-Said *et al*., 2016). Since organic materials are rich in ionized functional groups that adsorb heavy metals and keep them away from the active sites of the enzyme and reduce their inhibition of enzymatic activity.

Table (5) has been indicated that the interaction between the treatments and heavy metals had a significant effect on the activity of the enzyme L-glutaminase. This may be due to that fungi represent an important source in the production of most enzymes (Sparks, 2005; Hongjiang *et al*., 2014). The soil contaminated with zinc and inoculated with fungus have been recorded the highest activity of the enzyme (66.63 µg NH_4^{+}-N g^{-1} $2hr^{-1}$) compared to the soil contaminated with heavy metals and treated biologically and organically. This is consistent with the results of (Tables 2, 3 and Figure 3B). It showed an increase in the total number of fungi and the amount of released CO_2. The largest removal rate of heavy metals when inoculating zinc-contaminated soil with *A. niger* fungus, all of which are positive indicators for increase the activity of soil enzymes, including L-glutaminase.

Table 5. The interaction effect between treatments and heavy metals contamination on L-glutaminase activity (µg NH^{+}_{4}- N g^{-1} $2hr^{-1}$).

Sources	Zn	Cd	Pb	Ch.
Ch.	30.41	38.30	19.96	60.04
A. niger	66.63	64.21	60.66	90.12
H.A	46.93	52.05	48.33	80.76
O.M	40.60	48.41	36.02	70.83
RLSD 0.05= 2.9				

***Conclusion*:** Heavy metals (Cd, Pb and Zn) significantly inhibited the count of fungi, CO_2 evolved and L-glutaminase acclivity in soil. In contrast, treating soil with *Aspergillus niger*, cow manure or humic acid as a biological or organic amendments increased the mentioned parameters in soil contaminated with heavy metals. This indicates enhanced C and other nutrients benefit for fungi and other microorganisms in soil. Therefore, inoculated soil with 50× 10^3 cfu of *A. niger* , mixed with 4% cow manure, or 20 L ha^{-1} humic acid may be promising in biological activity of soil, then mitigated the harmful of heavy metals. However, these effects need to be studied in field conditions in presence of plant stands.

***Authors' Contributions statement*:** All authors' have same rule in paper preparation

***Conflict of interest*:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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